



Transcript: "Response to Bioterrorism: Agents of Bioterrorism"

(Editor's note: this script accompanies a presentation given in 1999—before the anthrax attacks of October 2001.)

The following is a presentation made by Harvey Holmes, Ph.D., Chief, Diagnostic Microbiology Section, Centers for Disease Control and Prevention. The accompanying PowerPoint slides are referred to in the text by the number appearing in the lower left corner of the slide.

1. Objectives

Following this presentation you should be able to

- 1.1 Apply appropriate biosafety practices when working with potentially dangerous organisms.
- 1.2 Recognize agents that could be used for bioterrorism and perform selected tests to rule them out.
- 1.3 Refer potentially dangerous specimens or organisms to an appropriate laboratory for confirmation.
- 1.4 Develop an emergency preparedness plan for your laboratory.

2. Introduction

As part of developing your emergency preparedness plan, each clinical laboratory should perform a risk assessment as described in the 4th edition of the manual *Biosafety in Microbiological and Biomedical Laboratories*. On page 76, you will find a "how-to" section for identifying procedures and methods that have the potential to produce aerosols, a primary source of laboratory-acquired infections. Also included are recommendations for how to eliminate such procedures or at least minimize the risk of producing an aerosol. As suggested in the manual, those aerosol-producing methods you cannot eliminate should be performed in a biosafety cabinet. You can either purchase a copy of this document or access it on the Internet at <http://www.cdc.gov/od/ohs/biosfty/biosfty.htm>. The fact that some virulent organisms can be transmitted through aerosols makes them agents of choice for bioterrorists.

This material is taken from a script that is available as a webcast on the CDC Public Health Emergency Preparedness and Response website (<http://www.bt.cdc.gov>). Several tables are also available at that website. The tables have detailed information on specimen collection, transport and processing, specimen exposure risk, biosafety levels required to work with each of the agents covered in this program, a summary of available vaccines, and charts with biochemical reactions and other identification information. You might want to download and print some of the tables on this website for quick reference material to use as you work at the bench.



3. Biosafety

As defined by the Laboratory Response Network (LRN), microbiology laboratories that might respond to a bioterrorism event are assigned to different levels based on their testing capabilities and safety capacity. The material in this lecture is primarily for the Level A laboratory. The Level A laboratory must be capable of performing the tests described in the specific agent protocols to “rule out or refer” and must meet the requirements of Biosafety Level (BSL) 2.

The BSL-1 laboratory works with well-characterized agents not known to consistently cause disease in healthy adults. An example would be a college laboratory working with *Bacillus subtilis*, a non-pathogen.

The BSL-2 laboratory works with known pathogens, such as *Salmonella*, *Shigella*, and *Campylobacter*. An example would be a hospital-based clinical laboratory. In addition to following BSL-1 requirements, laboratories that examine patient specimens must be directed by competent scientists, and personnel must be specifically trained in handling these organisms.

The BSL-3 laboratory works with known pathogens, including those that have the potential for aerosol transmission, such as tularemia, *Brucella*, or multiple-drug-resistant *Mycobacterium tuberculosis*. In addition to following BSL-2 requirements, these laboratories must decontaminate all wastes before disposal and all laboratory coats before laundering. Access to the laboratory is controlled, and baseline serum samples for employees may be required. Exhausted air is not recirculated and the laboratory is under negative pressure.

The Level A laboratory must operate under BSL-2 conditions. The key element that separates a BSL-2 from a BSL-1 laboratory is the ability to contain infectious aerosols, usually with a Class II biological safety cabinet (BSC). There are several types of Class II BSCs that can be used in a clinical laboratory, the most common type being the Class IIA. This cabinet is designed to recirculate 30% of the air drawn into the cabinet back into the laboratory. Class IIB cabinets are hard-ducted—that is, no air is allowed to be recirculated. Because this air is vented outside of the laboratory, small amounts of volatile or toxic chemicals may be manipulated under the Class IIB cabinet.

In addition to a properly operating safety cabinet, laboratories designated BSL-2 or higher must also make appropriate personal protective equipment available to all employees.

4. Identifying the Agents of Bioterrorism: Guidelines for Clinical (Level A) Laboratories

The role of a Level A laboratory is to rule out and refer. For example, if a small gram-negative coccobacillus (GNCB) is recovered from a blood culture bottle, a simple test that would rule out *Francisella tularensis*, *Brucella* spp., and *Yersinia pestis* would be a wet mount for motility. It is strongly recommended to do blood culture manipulations under the BSC to contain any aerosols.



If the isolate is motile, then it is very unlikely to be any of these agents. If, however, the isolate is nonmotile, then you might consider inoculating a urea slant to rule out *Brucella* spp. and adding a buffered-cysteine-yeast-extract (BCYE) to rule out *Francisella tularensis*. Keep in mind that with the capacity to genetically modify organisms today, there is always the possibility of a "designer" organism that was modified to enhance virulence or mask its identity.

What is the historical risk of acquiring these agents while working in the laboratory?

For anthrax, there have been no laboratory-associated cases since the late 1950s, when the anthrax vaccine was introduced. The overall incidence of anthrax in the United States is very low. Only 3-5 cases have been reported over the past 10-15 years, and those were primarily from the textile industry.

There have been only 3 cases of *Yersinia pestis* since the mid-1930s. The prevalence in the United States is 10-15 cases per year.

In contrast, *Brucella* spp. and *F. tularensis* are the most frequently acquired laboratory-associated infections. *Brucella* is the most commonly reported laboratory-acquired infection, and *Francisella* is third. The prevalence of *Brucella* is 100 cases per year, while 150-200 cases of *Francisella* are seen annually.

Only one documented case of botulinum toxin has been reported, but there are 100 cases of botulism each year.

In the past 20 years, there have been no smallpox cases in the world. The last case was laboratory acquired, and it occurred in 1978.

Viral hemorrhagic fevers, not considered endemic to the United States, are reported primarily in Africa.

Francisella tularensis

Francisella tularensis is the organism that causes tularemia. It can be considered an American disease. It was discovered in America and first studied by Americans. In 1910, George McCoy was assigned by the U.S. Public Health Service to investigate the deaths of a large number of ground squirrels in central California. At the same time, an outbreak of human plague was occurring in San Francisco and was believed to be exacerbated by the Great Quake of 1906. For this reason, the death of the squirrels was believed to indicate the spread of this epidemic to central California.

By 1912, McCoy, assisted by Chapman, isolated a bacterium on a specialized coagulated egg yolk medium and named it *Bacterium tularense*, after the county of origin, Tulare County, that was covered by a large variety of a bulrush reed known as tule.



This organism differed from the plague bacillus in that it was not readily seen in stained smears from infected organs of guinea pigs and it was nutritionally fastidious. In 1914, Vail described the first human case, an ocular infection, and, in that same year, Wherry and Lamb implicated the tick as the vector.

In 1921, Francis coined the term "tularemia" after describing the first human case of the septic or typhoidal form. He also implicated the deerfly as another vector. Eventually, the genus was named in his honor. Slide 9 shows other names and forms of the disease found in different locations.

In 1924, Ohara in Japan reported a milder form of the disease that we now know as the type B form. It differs from the more virulent form found in North America in a number of obscure biochemical reactions: type A ferments glycerol and sucrose and is citrulline-ureidase positive, whereas type B is negative for those tests. Ohara is also given credit for conducting the first human transmission studies. He placed heart blood of an infected rabbit onto the skin of his wife. She developed a self-limiting case of ulceroglandular tularemia with lesions from which he isolated the organism. He also isolated the organism from one of her lymph nodes. There are approximately 150-200 cases of tularemia annually in the United States, most commonly found in Arkansas and Missouri. In 1927, tularemia was placed on the Reportable Diseases List. It was removed in 1996, but CDC has subsequently requested that it be placed back on the list.

Tularemia is not transmissible from person to person, but can be acquired in the laboratory, especially when accidentally aerosolized. It is one of the most frequently reported laboratory-acquired infections. *Brucella* leads the list, followed by typhoid fever, then tularemia, *Mycobacteria tuberculosis*, and hepatitis B.

Tularemia has an extremely low infective dose of 10-50 organisms and a short incubation period averaging 3-5 days. Mortality is low if treated. The organism can remain viable in the soil for months. A vaccine is available and confers about 80% protection against aerosol exposure.

The tests that a Level A laboratory would be expected to perform if it received a specimen to rule out tularemia are a Gram stain and an evaluation of growth characteristics in both broth and agar.

Slide 13 is a Gram stain of *F. tularensis*. Notice that it is a small, gram-negative, poorly staining coccobacillus.

Francisella has typical growth characteristics in broth and agar. First, it is nutritionally very fastidious, requiring cysteine for robust growth. Cysteine heart agar (CHA) is an ideal isolation medium when enriched with chocolateized 9% sheep blood. Culturing for this organism is not part of Level A laboratory procedures, but you could encounter it when processing a routine clinical specimen from an infected patient. Although faint growth can be seen on primary culture sheep blood or chocolate agar incubated at 35° C, growth on these media will be lost on subculture.



This photograph on Slide 14 shows 3 plates showing *F. tularensis* growing at 72 hours. Although some growth may occur at 24 hours, individual colonies are too small to see. At 48 hours, colonies on sheep blood are gray-white with no hemolysis.

The plate on the left shows growth on CHA. Note the greenish color of the colonies. The middle plate shows the poor growth on sheep blood agar. On the right you can see the growth on chocolate agar at 72 hours—colonies are flat and gray with no sheen.

Other organisms described as gram-negative coccobacilli (GNCB) might also be isolated in the clinical laboratory. Organisms that would need to be differentiated from *Francisella* are listed on Slide 15. The list is not in any particular order of frequency. Some organisms, such as *Acinetobacter* and *Pasteurella*, are most likely to be encountered, while others such as DF (dysgonic fermenter)-3, are least likely to be seen. To help you separate these organisms from *Francisella tularensis*, *Yersinia pestis*, and *Brucella* spp., several tables that compare characteristics of these organisms can be viewed on the CDC website at <http://www.bt.cdc.gov/roleofclinlab.asp>.

Slide 17 summarizes the key features of *Francisella*. If you see tiny GNCB in a suspect specimen and the organism grows slowly on chocolate agar and poorly on blood agar but grows well on BCYE, then **refer** the isolate to a Level B laboratory.

Yersinia pestis

In the year 1347, approximately 30-40% (17-20 million) of the entire European population succumbed to the plague, also known as the Black Death. Plague is currently present on every major inhabited continent, except Australia. In 1899, it was introduced into the United States via San Francisco. Currently plague, along with yellow fever and cholera, are the only three diseases that have worldwide quarantine sanctions.

Natural transmission of the organism *Yersinia pestis* is through the bite of infected fleas. Of the approximately 1,500 species of fleas, only 30 or so are proven vectors, with oriental rat flea being the most competent. The average life span of an uninfected flea is about 6 weeks, though some may live as long as a year under certain conditions. A plague-infected flea lives only about 17 days because it dies from starvation and dehydration.

On average, there are 10-15 cases of plague per year in the United States, where it is primarily a disease of Native Americans in the Southwest. Several of the more recent human cases of pneumonic plague have occurred among veterinarians treating infected cats. Cats can acquire plague by ingesting plague-infected rodents. Of the cats that subsequently become ill, 92% have been shown to have the organism in their oral cavities.

There are three clinical forms of plague:

- **bubonic**—infection of lymph nodes;
- **septicemic**—bloodstream infection, the deadliest form; and
- **pneumonic**—infection that is transmitted through aerosols.



On Slide 21 are the recommended “specimens of choice” that one would collect from patients presenting with the various forms of plague. The best specimens for recovering *Y. pestis* in the pneumonic form of the disease are sputum and bronchial washings. A note of caution: make sure bronchial washings are collected within containment or isolation facilities. Among patients with bubonic plague, more than 80% will be blood-culture positive.

If you suspect *Y. pestis*, staining a smear of direct specimen material might be helpful. A Wayson stain enhances the bipolar (or “safety-pin”) appearance of the organism, especially in specimen material. The direct fluorescence antibody (DFA) assay from direct specimen material would be done by Level B laboratories. If you grow a suspected organism, CDC subject matter experts recommend the tests listed on Slide 23 to “rule out or refer” *Y. pestis*.

In a clinical laboratory, this organism would be processed like any other oxidase-negative, gram-negative rod—including routine gram-negative identification and susceptibility tests. *Y. pestis* can be identified by most commercial identification systems, but when this result is generated, you must carefully review all information available to decide if the identification is credible.

Yersinia are small, gram-negative coccobacilli that show bipolar staining, especially in older cultures. In the Wayson stain on Slide 25, you see pink-blue cells with a closed “safety-pin” look. The Wayson stain alone is not diagnostic, since other organisms such as *Pasteurella* also show bipolar staining. Bipolar staining can also be seen in the Gram stain. Confirmation of this organism will be performed by Level B laboratories using methods such as DFA.

On Slide 26, you can see the growth pattern of *Y. pestis* in brain heart infusion broth. To see this characteristic growth, incubate the tubes after inoculation at 28° C for 24-48 hours. Do not shake the tubes or you will disturb the typical growth pattern of *Y. pestis*. As you can see in the tube on the left, the broth of *Y. pestis* remains clear with clumps on the side and bottom. *Y. pseudotuberculosis*, in contrast, is cloudy throughout the tube.

Growth of *Y. pestis* on blood agar is similar to that of other enterobacteria. But when allowed to grow for 72 hours, you see the typical “fried egg” colonies shown on Slide 28.

Here on Slide 27 are some technical hints to help you rule out *Y. pestis*. Keep in mind that *Yersinia pestis* is an *Enterobacteriaceae* and, like all other members of that order, is gram-negative, oxidase-negative, ferment glucose and reduce nitrates to nitrites. *Yersinia* appear as definite rods that may show bipolar staining properties, especially when observed in a clinical specimen or older culture.

If you culture an organism possessing these characteristics from more than one patient in a short period of time, **refer**.

Smallpox (*Variola Virus*) and Viral Hemorrhagic Fever



In suspect cases of either smallpox or the agents of viral hemorrhagic fever, usually suspected based on clinical presentation, you will contact your state department of health, who will then notify the CDC.

Smallpox

Some historians claim that smallpox has killed more people than any other disease. The estimate of deaths in the 20th century alone is more than 300 million. In 1966, the World Health Organization announced its goal to eradicate smallpox in the world, and in 1971, routine vaccination in the United States was discontinued.

This photograph shows the last case of variola major, which occurred on an island off of Bangladesh in 1976. Here we see the child with smallpox and after he recovered.

In 1977, the last case of variola minor occurred in Somalia. Here is a picture on Slide 31 of the last case, a young man from Somalia who was only 23 years old when this photograph was taken. Fortunately, he recovered. Ten months later, a medical photographer whose office was adjacent to a virology research laboratory, acquired smallpox and died. This was a laboratory-acquired case—the last known case—and in 1980, smallpox was declared eradicated. In 1982, the vaccine was no longer required for international travel, and in 1983, the vaccine was no longer available for any civilian vaccinations.

Variola virus is a large, complex virus measuring 300 nanometers in length and is easily distinguished from *Herpes simplex virus* and *Varicella zoster* by electron microscopy. *Variola virus* is a large, DNA virus with a dumbbell-shaped core and a complex membrane system (see Slide 32). *Vaccinia virus*, a related virus used for the vaccine has similar morphology in electron micrographs.

The key responsibility for the Level A laboratory is to rule out chickenpox and then to obtain instructions to properly collect vesicular fluid. However, **DO NOT COLLECT THE SPECIMEN UNTIL YOU CONTACT YOUR STATE LABORATORY or CDC for instructions.**

Agents of Viral Hemorrhagic Fever (VHF)

These are considered by some experts to be the most lethal agents known to man. Ebola has a mortality rate approaching 88%, and Marburg a 25% mortality rate. To date, the reservoirs of these two agents are unknown. There are four families that contain the different hemorrhagic fever viruses: *Filoviridae*, *Arenaviridae*, *Bunyaviridae*, and *Flaviviridae*.

Ebola and Marburg are both in the *Filoviridae* family, while Lassa fever is in the *Arenaviridae* family. As you can see on Slides 35-39, the viruses are responsible for some of the most serious viral diseases of man.

Person-to-person transmission of Lassa, Ebola, and Marburg occurs through direct contact with infectious body fluids such as blood, urine, semen, and saliva. The infective doses of these viral



groups are extremely low. Incubation periods range from 4-21 days, and the illnesses last from 7-16 days. Mortality rates vary, approaching 90% for Ebola. These organisms are not stable and fortunately do not occur naturally in the United States. Unfortunately, no vaccine is available at this time for any of these viruses.

Since the diagnosis for VHF is a clinical decision, the role of your laboratory will be to contact your respective state health laboratory for assistance, and they will in turn notify CDC. No specimen will be accepted without proper consultation. For the complete list of specimens, refer to the CDC Public Health Emergency Preparedness and Response website (<http://www.bt.cdc.gov>). As you can see on Slide 42, shipping and handling requirements depend on the specimen you are sending. Contact CDC or your state health laboratory if you have any questions or do not have access to the website.

***Brucella* spp.**

These organisms are small, faintly staining, gram-negative coccobacilli. The organism will most likely be recovered from the blood or bone marrow of an infected patient—generally only after prolonged incubation. All of the *Brucellae* are oxidase-positive, urea-positive, catalase-positive, and glucose-oxidizers. The commercial identification systems available at this time will code out the unknown organism as “unidentified.”

An organism that is isolated from blood or bone marrow and is consistent with *Brucella* must be handled carefully. *Brucella* is the most commonly reported laboratory-associated bacterial infection. The common practice of poking or sniffing a culture plate is a dangerous practice and should be avoided. There is no vaccine, so the only barrier between the laboratorian and *Brucella* is good, safe technique.

Brucellosis is a zoonotic disease caused by four species of *Brucella*. In humans, *B. melitensis* produces the most severe disease, followed by *B. suis*, which typically has associated skeletal involvement. *B. abortus* and *B. canis* produce a milder but more insidious chronic form of the disease.

In the United States, approximately 100 cases are reported each year. Most of the cases are from California and Texas, and about one half are associated with some aspect of the meat processing industry. It is likely that the disease is grossly underreported—for every reported case, it is estimated that 20-25 go unreported.

In 1887, David Bruce isolated an organism from the spleens of dying British soldiers on the island of Malta. Because it appeared to be a coccus, he named it *Micrococcus melitensis*. For several decades, its bacillary nature went unrecognized. Bernard Bang, in 1897, reported on a bovine strain from Denmark, naming it *Bacillus abortus*; Jacob Traum, in 1914, discovered *Brucella suis* from an aborted swine fetus; and finally, Leland Carmicheal, a virologist, isolated the canine strain in 1968. The relationship among these organisms was first recognized in 1920 by Alice Evans of the U.S. Department of Agriculture. Her work was corroborated by Meyer and Shaw, who proposed the genus name *Brucella*. In 1954, the United States “weaponized”



B. suis. This is believed to be the first U.S. biological weapon.

Slide 47 shows the various modes of transmission of *Brucella*: ingestion, skin penetration, and inhalation. Ingestion, the most common form, is usually the result of consuming unpasteurized dairy products. Aerosols of *Brucella* are highly infectious, making inhalation the mode that could be used in bioterrorism. Aerosols can also infect the conjunctiva.

The infective dose of *Brucella* is fewer than 100 organisms, and the average incubation period is 5 days to 6 months. For laboratory-acquired brucellosis, the incubation period is only 30 days.

The disease can last for years—in some cases as long as 20 years.

Brucellosis is characterized by fever, malaise, headache, and muscle pain; the hallmark of the disease is the intermittent, irregular fever of variable duration complemented by severe fatigue. Bacteria in humans produce a “generalized” infection localizing in the reticuloendothelial system—especially in the spleen and bone marrow. In the animal host, the bacteria are found in large numbers within the genital tissue, udders, uterus, and epididymis. A common manifestation in men infected with this organism is swollen testicles accompanied by profound depression.

Person-to-person transmission does not occur as a rule, but there has been a report of sexual transmission between a laboratorian and his spouse.

The mortality rate is low—most fatal cases are associated with underlying endocarditis.

Brucella survives in urine for 6 days, in dust for 6 weeks, and in soil and water for 10 weeks. Several studies have shown that 20-50% of individuals suffering from brucellosis shed the organism in their urine at some time during their course of infection.

The diagnostic method of choice is serological testing of serum. IgG appears within weeks of infection and a single titer of 1:160, or a four-fold increase in titer, accompanied by symptoms consistent with brucellosis is diagnostic.

The best way to recover *Brucella* is to culture blood or bone marrow specimens. The newer “continuous-read” blood culture instruments have been reported to recover *Brucella* in less than 5 days. However, at this time, CDC continues to recommend holding the culture for at least 21 days. Other specimens for recovery are spleen and liver tissue post-mortem.

Here are the tests that a Level A laboratory would be expected to perform: examination of the colonial morphology on sheep blood agar, Gram stain, oxidase, and urea hydrolysis.

Remember, *Brucella* are considered BSL-3 agents when cultures are being manipulated. Unfortunately, most *Brucella* cultures go unrecognized unless there are several simultaneous cases.



Brucella are tiny, faintly staining, gram-negative coccobacilli. Because they are nutritionally very fastidious, you may have no visible growth for 48-72 hours. On blood agar, colonies are small, convex, nonhemolytic, and nonpigmented.

Oxidase and urease tests can help differentiate *Brucella* species. Three of the *Brucella* species are consistently oxidase-positive. *B. suis* and *B. canis* will hydrolyze a urea slant rapidly, whereas *B. abortus* and *B. melitensis* may take overnight.

If you see a small GNCB in a blood culture, you might consider performing a quick wet-mount and looking for motility. If it is nonmotile, it could be *Brucella*. Inoculate a urea slant and do an oxidase test. Other organisms that share these characteristics include *Ochrobactrum anthropi* (formerly Vd) and *Agrobacterium* spp. Motility test is an easy way to separate *Brucella* from these two organisms—*Brucella* is nonmotile, whereas the other two are motile. Other characteristics can be found on the website mentioned earlier. *Brucella* has been reportedly misidentified as *Moraxella*, *Haemophilus*, and even “slow-growing” staphylococci.

Slides 52 and 53 summarize the key tests. The take-home lesson: **refer**.

Clostridium botulinum

Clostridium botulinum was first described in 1897, following a foodborne outbreak in Belgium. It produces a toxin that is among the most potent neurotoxins known. The toxin is 100,000 times more toxic than sarin, a well-known organophosphate nerve agent. If this organism is suspected, the clinical laboratory's role is to properly package and ship collected specimens.

The disease botulism is diagnosed clinically based on the patient's physical findings and case history. Clinical laboratories should collect specimens and immediately notify their state health departments. The map on Slide 59 shows which states currently have the capacity to test for botulinum toxin. Actually, New York and California each have two laboratories that can perform these tests.

There are 7 types of botulinum toxin, designated Types A through G. Types A, B, and E are the most common types encountered in human disease, with rare cases caused by Type F. Types C and D have been detected in birds and some mammals. Type G, identified in 1970, has yet to be incriminated in humans or animals. The infective dose of Type A toxin is 1 nanogram, meaning that slightly over 20 grams of purified toxin administered to the entire population of the United States would result in half of them dying. Onset occurs only 18-36 hours after exposure. Typical clinical signs include dry mouth, dilated pupils, and a progressive muscle weakness leading to respiratory failure and death.

In foodborne outbreaks, mortality of the first case may be as high as 25%, with subsequent cases about 4%. Slide 61 shows the sensitivity of various laboratory tests that are available to confirm the diagnosis of foodborne botulism. Of 309 clinically diagnosed individuals, only 51% had stool cultures positive for the organism, 37% had positive toxin in their sera, and only 23% had detectable toxin in their stool samples. When all three testing methods were combined, the



overall sensitivity was only 65%. Prior to the shipment of any botulism-associated specimen, the designated receiving laboratory must be notified and approved by the state health department.

Slide 63 shows a list of the recommended specimens that can be used to confirm the diagnosis of botulism, but as we've seen, even with these tests, the toxin won't always be detected. Remember to exercise extreme caution when handling materials suspected of containing these toxins.

Bottom line: **refer**.

Bacillus anthracis

Anthrax is a disease of antiquity—the fifth plague of the Bible, referred to found in Exodus 9:3, may have been one of the earliest descriptions of an anthrax outbreak. During the 17th century, a disease known throughout the Middle East and Europe as the Black Bane was likely anthrax. In 1877, Koch reported anthrax as the first disease proven to be caused by a specific bacterium. In 1881, Louis Pasteur successfully attenuated *B. anthracis*, creating the first bacterium for which immunization was available.

Anthrax is a zoonosis, causing disease in both domesticated and wild herbivores that ingest spores from the soil. Humans are actually an accidental host. Anthrax has a worldwide distribution with high-risk areas in Africa, Asia, and the Middle East.

Infection in humans occurs after exposure to spores from infected animals or their products or a deliberate release in a bioterrorism event. There are three forms of anthrax: **cutaneous**, **gastrointestinal (GI)**, and **inhalational**.

Cutaneous anthrax is the form most commonly encountered, whereas gastrointestinal is the rarest form. GI anthrax occurs following the consumption of tainted meat. Inhalational anthrax, sometimes referred to as woolsorter's disease, is occasionally contracted by people who work with sheep wool. Inhalational anthrax is also the most serious outcome of an intentional aerosol release of this organism in a bioterrorism event.

In a smear prepared from a specimen taken from an early cutaneous vesicle (see Slide 67), we would most likely observe the large, gram-positive bacilli of anthrax (see Slide 77). A specimen from an eschar would be less sensitive. Both lesions, however, are appropriate specimens for culture. (These photographs are used here courtesy of the World Health Organization and Dr. Peter Turnbull. They were obtained from the World Health Organization website at <http://www.who.int/emc>. This website has one of the most comprehensive overviews of anthrax currently available)

B. anthracis produces three plasmid-coded virulence factors: an antiphagocytic capsule and at least three proteins that combine to form two toxins. These proteins, known as lethal factor, edema factor, and protective antigen, are found on one plasmid; a second plasmid codes for the peptide capsule consisting of poly D-glutamic acid. Anthrax spores, which measure between 1.5



and 3 microns, lend themselves very well to aerosolization. The infective dose is believed to be 8,000 to 15,000 spores.

Inhalational anthrax generally has an incubation period of 1-6 days. However, during the Sverdlovsk release, cases appeared as late as 43 days after the initial event, suggesting the possibility of secondary environmental exposure. Anthrax spores have been known to survive for decades under the proper environmental conditions.

The pathogenesis of anthrax, including the signs, symptoms, and disease development, has been covered in detail in another videotape in this series titled the Clinical Aspects of Critical Biological Agents (see <http://www.bt.cdc.gov/roleofclinlab.asp>). Briefly, after the incubation period, a nonspecific flu-like illness develops characterized by fever, muscle aches, headache, and a nonproductive cough with mild chest discomfort. This phase is followed by a short period of improvement of 1-3 days culminating in acute respiratory collapse and death. Late in the course of infection, a direct Gram stain of peripheral blood may reveal broad gram-positive rods.

Anthrax has been shown to have little potential for person-to-person transmission. Infected tissue contains only vegetative encapsulated bacilli. Typically, spore-formation does not occur in tissue but only when bacilli are exposed to air or grown in a non-CO₂ atmosphere.

In inhalational anthrax, a chest X-ray is often pathognomonic, revealing a widening of the mediastinum with pleural effusions without infiltrates. The photomicrograph of a mediastinal lymph node seen on Slide 71 contains microcolonies of *B. anthracis*, visible as small blue clumps in the center of the photograph. (This image was downloaded from the CDC Public Health Image Library website at <http://phil.cdc.gov/Phil>.)

While the early recognition of anthrax is likely to require a heightened suspicion, the diagnosis will be supported by presence of large, gram-positive bacilli from skin lesions or in direct blood smears. In the event of an announced bioterrorist event, a large number of gram-positive bacilli cultured from swabs of the nares might also support such a suspicion. The recovery rate from sputum will be very low because inhalational anthrax is a mediastinitis and not a true pneumonia.

The chance of recovery of the organism from stool is rare.

Next to the cutaneous forms, recovery from blood is the most likely.

Here are the key tests to rule out *B. anthracis*: a Gram stain, growth characteristics on sheep blood agar, lack of motility, and its ability to sporulate aerobically.

Two tests that are optional but possibly helpful are the penicillin inhibition test and capsule formation. A penicillin-inhibition zone test in which a 10-unit penicillin disk is dropped on a sheep blood agar plate after inoculation of a suspension of the suspect organism. The bacterial suspension, made up of 2-3 colonies suspended in half a milliliter of sterile distilled water, is added to the plate and incubated overnight at 35-37° C in a non-CO₂ incubator. Zone sizes



greater than 15 mm are considered suspicious for *B. anthracis*, and most strains actually have zone sizes greater than 20 mm. Remember, this is not a susceptibility test.

Capsule formation, another optional Level A test, can be demonstrated by growing cells on nutrient agar in the presence of 5% CO₂ or on other basal media supplemented with 0.8% sodium bicarbonate. Virulent strains will give rise to heavily encapsulated bacilli.

On sheep blood agar, *B. anthracis* produces nonhemolytic, flat or slightly convex, irregularly shaped, circumscribed colonies. Well-isolated colonies typically measure 2-3 mm after 18-24 hours incubation. The colonies have a ground-glass appearance, and the edges may be slightly undulate with curly tailing projections, as seen on Slide 74. The projections may become quite pronounced in certain strains, especially in older colonies. These have been referred to as "Medusa Head" colonies. Another helpful characteristic is the tenacity of the colony itself. When grown on sheep blood agar and teased with a loop, the colony growth will stand up like beaten egg white (see Slide 76).

A Gram stain of growth from a colony at 35° C in a non-CO₂ incubator is seen in Slide 77. *B. anthracis* is a large, gram-positive rod measuring 1-1.5 microns by 3-5 microns. Spore formation readily occurs within 24-30 hours on sheep blood agar incubated in a non-CO₂ atmosphere. Oval subterminal spores are produced with little or no swelling of the vegetative cell.

In reviewing the key tests that will rule out *B. anthracis* from other *Bacillus* spp., common characteristics among all the species of *Bacillus* are gram-positive, rods, catalase-positive, will grow both aerobically or anaerobically, but form spores aerobically. *Bacillus* spp. that form oval spores that do not significantly swell the vegetative cell and have a ground glass colonial morphology would fall into the *Bacillus* Morphology Group 1 that includes *anthracis*, *cereus* var. *mycoides*, and *thuringiensis*.

Within this Group 1, there are two species that are consistently nonmotile: *B. anthracis* and *B. cereus* var. *mycoides*. *Bacillus* spp. in Group 1 that are nonmotile and show no hemolysis on sheep blood agar should be considered presumptive *anthracis* and be immediately referred to a Level B laboratory for final identification. Over the past 10 years (as of Fall 2000), the most common *Bacillus* spp. submitted to CDC to rule out *B. anthracis* were nonmotile *B. megaterium*.

Lastly, a *Bacillus* spp. recovered from a set of blood culture bottles after 10 hours of incubation indicates a high initial inoculum, not consistent with a "contaminant," and should motivate the laboratorian to check on the status of the patient. Overwhelming sepsis caused by an organism as virulent as *B. anthracis* can quickly cause the death of the patient.

5. Summary

In summary, we have talked about the organisms that are the most dangerous and likely to be used in a bioterrorist event.

Always remember:



- Know when to hold and when to fold—don't waste time if you suspect a bioterrorist agent—refer it.
- Use proper biosafety techniques at all times.
- Refer suspicious organisms.
- Be familiar with the workings of the Laboratory Response Network (LRN).
- You might be the key person who helps contain a potentially dangerous situation.